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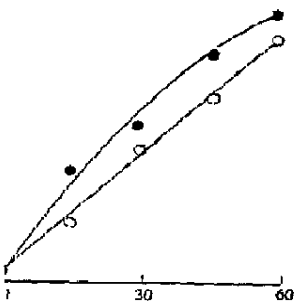

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ported previously[45–47] that the 670,000 proteasome complex (previously called macroxyproteinase or MOP in our laboratory) is largely responsible for the degradation of oxidatively modified proteins in red blood cells, and, in our experimental model, it enhanced the release of dityrosine. Therefore, dityrosine release can be used as a marker for the selective proteolysis of oxidatively modified proteins.[19,20]

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[40] Quantitation of 4-Hydroxynonenal Protein Adducts

By KOJI UCHIDA and EARL R. STADTMAN

Introduction

Lipid peroxidation has been associated with important pathophysiological events in a variety of diseases, drug toxicities, and traumatic or ischemic injuries. It has been postulated that free radicals and aldehydes generated during the process may be responsible for these effects because of their ability to damage cellular membrane, protein, and DNA. Studies have shown that the cytotoxicity of products of lipid peroxidation is due in part to the formation of α,β-unsaturated aldehydes, especially 4-hydroxy-2-alkenals.[1,2] 4-Hydroxy-2-alkenals elicit a variety of cytopathological effects including inactivation of enzymes,[1] lysis of erythrocytes,[2] chemotactic activity toward neutrophils,[3] and inhibition of protein and DNA synthesis.[4] Among the aldehydes formed, 4-hydroxynonenal is the major product of lipid peroxidation, and it has been suggested to play a major role in liver toxicity associated with lipid peroxidation.[2,5–7]

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It is generally accepted that 4-hydroxy-2-alkenals exert these effects because of their facile reactivity toward molecules with sulfhydryl groups.[8–10] The α,β-double bond of 4-hydroxy-2-alkenals reacts with sulfhydryl groups to form thioether adducts via a Michael-type addition. Whereas it was generally accepted that the aldehyde moiety of 4-hydroxynonenal and other 2-alkenals reacts with primary amino groups to form α,β-unsaturated aldimines,[11,12] it is now evident that the ϵ-amino group of lysine residues in proteins may also undergo Michael addition reactions with the α,β-double bond of 4-hydroxynonenal to form secondary amines possessing an aldehyde group.[13] Furthermore, the imidazole groups of histidine residues in proteins also undergo Michael addition type reactions.[14] Accordingly, it is not surprising that the modification of low-density lipoprotein (LDL)[15,16] and several other proteins[17] by 4-hydroxynonenal is associated with a significant loss of lysine and histidine residues. It is thus important to establish the procedures to detect proteins derived from covalent attachment of 4-hydroxynonenal to amino acid side chains in order to understand the mechanism of a large number of biological effects induced by 4-hydroxynonenal.

Quantitation of 4-Hydroxynonenal Protein Thioether Adducts

Taking advantage of the fact that Raney nickel catalyzes cleavage of thioether bonds,[18–21] a procedure has been developed[22] by which lipid-derived protein carbonyl derivatives can be distinguished from protein

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[22] K. Uchida and E. R. Stadtman, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5611 (1992).

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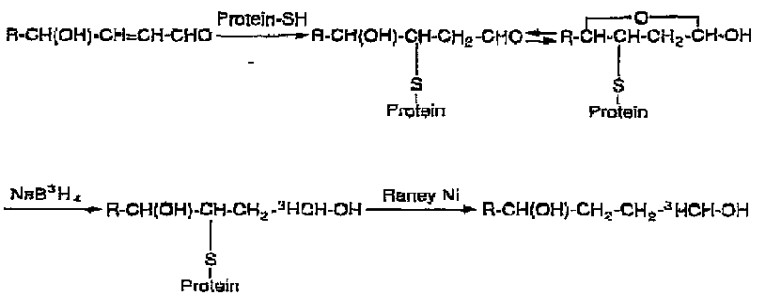

FIG. 1. Experimental scheme to determine 4-hydroxynonenal covalently attached to protein sulfhydryl groups by means of a thioether linkage. R is $CH_3(CH_2)_4$—.

carbonyl derivatives generated by other reactions including metal-catalyzed oxidation and glycation reactions.[23] The strategy used is illustrated in Fig. 1. In this procedure the aldehyde moiety of the 4-hydroxynonenal thioether adduct is reduced to the corresponding ^{3}H-labeled dihydroxyalkane by treatment with NaB^3H_4. The ^{3}H-labeled dihydroxyalkane is then released from the protein by cleavage (desulfurization) of the thioether bond by means of Raney nickel catalysis. The free ^{3}H-labeled dihydroxyalkane is then separated from the reaction mixture by extraction with an organic solvent and is quantitated by radioactivity measurement.

This method can be used to measure the amount of 4-hydroxynonenal that is conjugated to proteins by a thioether linkage only. It cannot be used to measure that fraction of 4-hydroxynonenal that is bound to proteins via both a thioether linkage and also by a Schiff base linkage, as would occur if the aldehyde moiety of the primary Michael addition product (thioether) undergoes a secondary reaction with the free amino group of lysine residues in the same or a different protein subunit. On reduction with NaB^3H_4, the Schiff base would be converted to a stable ^{3}H-labeled secondary amine. Subsequent treatment of the complex with Raney nickel will lead to cleavage of the thioether linkage, but the derivatized 4-hydroxynonenal would still be tethered to the protein via the secondary amine linkage and would therefore not be extractable by organic solvents.

Reagents and Equipment

4-Hydroxynonenal: A stock solution of *trans*-4-hydroxy-2-nonenal is prepared by acid treatment (1 m*M* HCl) of 4-hydroxynonenal

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diethylacetal and stored at −20°. The concentration of 4-hydroxynonenal stock solution is determined from the molar extinction coefficient of 4-hydroxynonenal at 224 nm (ε = 13,750). The purity of the stock solution should be checked by HPLC prior to the experiment.

NaB^3H_4, 100 m*M*, in NaOH, specific activity about 100 mCi/mmol: Add the required volumes of 1 *M* $NaBH_4$ and 0.1 *N* NaOH to the NaB^3H_4 and store at −20°.

Guanidine hydrochloride, 8 *M*, with 13 m*M* EDTA and 133 m*M* Tris, adjusted to pH 7.2 with HCl

Raney nickel-activated catalyst: Rinse thoroughly with water and ethanol prior to use. Avoid complete drying since Raney nickel is inflammable.

High-performance liquid chromatography (HPLC): Reversed-phase HPLC is performed on a Hewlett-Packard (Palo Alto, CA) Model 1090 chromatograph equipped with a Hewlett-Packard Model 1040A diode array UV detector.

Preparation of ^{3}H-Labeled 4-Hydroxynonenal Adducts of N-Acetylcysteine and Glutathione

To prepare standard samples of the 4-hydroxynonenal–protein adduct, 2 m*M* *N*-acetylcysteine or glutathione is incubated with 2 m*M* 4-hydroxynonenal in 1 ml of 50 m*M* sodium phosphate buffer (pH 7.2) at 37° for 2 hr. Formation of 4-hydroxynonenal–*N*-acetylcysteine and 4-hydroxynonenal–glutathione adducts can be followed by reversed-phase HPLC on an Apex Octadecyl 5U column (Jones Chromotography, Lakewood, CO): the adducts are eluted at 1 ml/min with a linear gradient of 0–100% (v/v) acetonitrile in 0.05% trifluoroacetic acid for 25 min. The elution profiles are monitored by the absorbance at 210 nm. Under these chromatographic conditions, the 4-hydroxynonenal–*N*-acetylcysteine and 4-hydroxynonenal–glutathione adducts are eluted at 10.7 and 11.0 min, respectively.[20]

Take an aliquot (400 μl) of the reaction mixture and add 10 m*M* EDTA (40 μl)/1 *M* NaOH (40 μl)/100 m*M* NaB^3H_4 (40 μl) to a 1.5-ml Sarstedt tube fitted with O ring and a cap. After incubation for 1 hr at 37°, terminate the reaction by adding 200 μl of 1 *N* HCl, allow to stand 5 min in the hood, and then load the mixture on a Sep-Pak C_{18} cartridge: elute with 5 ml water followed by 5 ml of methanol. Concentrate the methanol fraction in a vacuum centrifuge (Savant Instruments, Inc., Farmingdale, NY), redissolve in 200 μl of methanol, and subject to reversed-phase HPLC to purify the ^{3}H-labeled adducts. Collect the eluted fractions in 1-ml aliquots and monitor the amount of ^{3}H in each fraction by scintillation counting. Collect

the fractions that contain the ^{3}H-labeled products, concentrate, and then redissolve the adducts in 50 m*M* sodium phosphate buffer (pH 7.2). This solution should be stored at −20°.

Preparation of ^{3}H-Labeled 4-Hydroxynonenal-Modified Proteins

Incubate 1 mg protein with 2 m*M* 4-hydroxynonenal in 1 ml of 50 m*M* sodium phosphate buffer (pH 7.2) to prepare 4-hydroxynonenal-modified proteins. After incubation for 2 hr at 37°, take an aliquot (400 μl) of 4-hydroxynonenal-modified protein and add an equal volume of 20% trichloroacetic acid (w/v, final concentration). Centrifuge the tubes in a tabletop microcentrifuge at 11,000 *g* for 3 min at room temperature and discard the supernatant. Dissolve the precipitate with 400 μl of 8 *M* guanidine hydrochloride/13 m*M* EDTA/133 m*M* Tris (pH 7.2) and treat with 0.1 *M* EDTA (40 μl)/1 *N* NaOH (40 μl)/0.1 *M* NaB^3H_4 (40 μl) in a 1.5-ml Sarstedt tube fitted with O ring and a cap. After incubation for 1 hr at 37°, add 1 *N* HCl (100 μl) to terminate the reaction and then apply to the PD-10 column (Sephadex G-25, Pharmacia, Uppsala, Sweden), equilibrated in 6 *M* guanidine hydrochloride, in order to separate protein-bound counts from free radioactivity. Collect every 500 μl and determine protein recovery by spectrophotometry and radioactivity by liquid scintillation counting.

Raney Nickel Desulfurization

Take an aliquot (50 μl) of the ^{3}H-labeled 4-hydroxynonenal–*N*-acetylcysteine adduct, 4-hydroxynonenal–glutathione adduct, or 4-hydroxynonenal-modified proteins prepared above and mix with 400 mg of Raney nickel and 300 μl of 8 *M* guanidine hydrochloride/13 m*M* EDTA/133 m*M* Tris (pH 7.2) in a 1.5-ml Sarstedt tube fitted with O ring and a cap. After incubation for 15 hr at 55°, released products are extracted twice with 500 μl each of chloroform/methanol (9 : 1, v/v): add chloroform/methanol solution to the mixture, vortex vigorously, and centrifuge the tubes in a tabletop microcentrifuge at 11,000 *g* for 3 min. After centrifugation, collect the chloroform (lower) layer and add dehydrated sodium sulfate (50–100 mg) to the chloroform solution. After vortexing, take an aliquot (50 μl) of chloroform extract, mix with 5 ml of scintillation liquid, and count the radioactivity to measure the amount of released product.

Properties

On treatment with Raney nickel, the ^{3}H-labeled product can be recovered in 80–90% yield from both 4-hydroxynonenal–*N*-acetylcysteine and 4-hydroxynonenal–glutathione adducts in a solvent (10% methanol/chlo-

roform)-extractable form.[22] Before Raney nickel treatment, only 1 and 25% of the radioactivity in the *N*-acetylcysteine and glutathione adducts, respectively, were extracted by the solvent. It was considered that the small amount (25%) of solvent-extractable radioactivity present in the 4-hydroxynonenal–glutathione derivative before Raney nickel treatment is due to the contamination of the adduct preparation with radiolabeled reduced forms of free 4-hydroxynonenal.

When glyceraldehyde-3-phosphate dehydrogenase, which contains four sulfhydryl groups per subunit, was modified with 4-hydroxynonenal, 3.2 of these sulfhydryl groups are lost; however, 0.54 mol/mol of the labeled adduct was released in a solvent-extractable form on treatment with Raney nickel. Therefore, only 17% of the modified cysteine residues are present as simple 4-hydroxynonenal–thioether adducts. On HPLC analysis, ^{3}H-labeled product released after treatment with Raney nickel was indistinguishable from the products obtained following Raney nickel treatment of the 4-hydroxynonenal–*N*-acetylcysteine and 4-hydroxynonenal–glutathione adducts. Thus, the procedure using ^{3}H-labeling followed by Raney nickel treatment enables one to determine bona fide 4-hydroxynonenal–protein thioether adducts and attests to the fact that the reaction of 4-hydroxynonenal with cysteine residues of proteins may lead to derivatives other than simple thioether adducts (see Discussion).

Quantitation of 4-Hydroxynonenal–Histidine and 4-Hydroxynonenal–Lysine Adducts by High-Performance Liquid Chromatography

The oxidation of low-density lipoprotein (LDL) is accompanied by a loss of histidine and lysine residues[24,25] and conversion of LDL to a form that is taken up by macrophages, giving rise to foam cells which have been implicated in atherogenesis.[25] A role of 4-hydroxynonenal in LDL oxidation and its possible involvement in atherogenesis are underscored by the following observations: (1) 4-hydroxynonenal is formed during the oxidation of LDL,[12] and (2) treatment of unoxidized LDL with 4-hydroxynonenal leads to the modification of lysine and histidine residues,[15,16] and to conversion of LDL to a form that is taken up by the scavenger receptor on macrophages.[26] The development of procedures

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for the detection and quantitation of the 4-hydroxynonenal–lysine and histidine adducts in proteins is therefore fundamental to assessment of the role such adducts have in the cytotoxicity of lipid peroxidation and atherogenesis.

Sample Preparations

N-Acetylhistidine and *N*-acetyllysine are used to prepare standard samples of 4-hydroxynonenal–histidine and 4-hydroxynonenal–lysine adducts, respectively. Treat 50 mg of *N*-acetylhistidine (or *N*-acetyllysine) with 5–10 m*M* 4-hydroxynonenal in 2 ml of 50 m*M* sodium phosphate buffer (pH 7.2) for 20 hr at 37°. The products are isolated by reversed-phase HPLC, using a TSK-GEL ODS-80 TM column (0.46 × 25 cm) (Tosohaas, Philadelphia, PA) and a linear gradient of 0.05% trifluoroacetic acid in water (solvent A)–acetonitrile (solvent B) (time = 0, 100% A; 20 min, 0% A), at a flow rate of 1 ml/min. Under these chromatographic conditions, 4-hydroxynonenal adducts of *N*-acetylhistidine and *N*-acetyllysine are eluted at 11.2 and 10.8 min, respectively. 4-hydroxynonenal-modified proteins are prepared according to the procedures described above.

Acid Hydrolysis of 4-Hydroxynonenal-Modified Samples

An aliquot (0.1 ml) containing 0.1 mg of 4-hydroxynonenal-modified proteins or 1–10 nmol of purified 4-hydroxynonenal–*N*-acetylhistidine or 4-hydroxynonenal–*N*-acetyllysine adduct is placed in a hydrolysis vial. Add 10 μl each of 10 m*M* EDTA, 1 *N* NaOH, and 0.1 *M* $NaBH_4$, then incubate for 1 hr at 37°. Add 30 μl of 1 *N* HCl to terminate the reaction and concentrate in a vacuum centrifuge. Add 200 μl of 6 *N* HCl and flush the headspace with nitrogen for 60 sec, then cap using a screw cap fitted with a 12 mm Teflon/silicone liner with the Teflon facing the HCL. Place the vial in a benchtop heater at 110° and incubate for 20 hr. Then concentrate the mixture in a vacuum centrifuge and redissolve in the desired volume of 50 m*M* sodium phosphate buffer (pH 8.0) containing 1 m*M* EDTA.

Amino Acid Analysis

Reagents

o-Phthaldialdehyde (OPA): Dissolve 3.09 g boric acid in 40 ml HPLC grade water and adjust the pH to 10.4 with KOH pellets. Dissolve 134 mg OPA in 0.75 ml HPLC grade methanol in a Sarstedt type tube and then add to the borate. Add 0.21 ml 2-mercaptoethanol, water, and KOH to bring the pH back to 10.4 and the volume to

50 ml. Pipette into a 1.5-ml Sarstedt tube fitted with O ring and a cap and store at −20°.

Brij 35: Make 10 ml by mixing 250 μl of 2 *M* NaCl, 167 μl of 30% Brij 35, and water. Make this up freshly each day because the Brij is not stable.

Derivatization. Pipette 10 μl *o*-phthalaldehyde onto the bottom of a Sarstedt tube. Tilt the tube, place 10 μl of sample on the side of the tube, mix with a vortex, and start the timer. At 0.25 min, add 180 μl Brij 35. At 1 min, inject 100 μl and start the gradient.

Chromatographic Analysis. Reversed-phase HPLC is performed on a Hewlett-Packard Model 1090 chromatograph equipped with a Hewlett-Packard Model 1046A programmable fluorescence detector (excite at 340 nm and follow emission at 450 nm). The column we used to use is a 15 cm C_{18} column from Jones Chromatography (5 μm size) (Jones Chromotography, Lakewood, CO). Control the column temperature at 30°. The flow rate is 2.0 ml/min. The following gradient program is used. All gradients are linear (A, 100 m*M* NaCl; B, water; C, methanol).

0.0 min	A = 50.0%, B = 50.0%
0.3 min	A = 37.5%, B = 37.5%, C = 25.0%
7.0 min	A = 37.5%, B = 37.5%, C = 25.0%
11.0 min	A = 30.0%, B = 30.0%, C = 40.0%
12.0 min	A = 25.0%, B = 25.0%, C = 50.0%
16.0 min	A = 22.5%, B = 22.5%, C = 55.0%
18.0 min	A = 12.5%, B = 12.5%, C = 75.0%
20.0 min	A = 12.5%, B = 12.5%, C = 75.0%
21.0 min	A = 50.0%, B = 50.0%

Properties

Treatment of the 4-hydroxynonenal–histidine and 4-hydroxynonenal–lysine adducts with $NaBH_4$ stabilizes the histidyl-4-hydroxynonenal linkage. If the reduction step is omitted, acid hydrolysis of the 4-hydroxynonenal adducts leads to quantitative release of the histidyl and lysyl moieties as free amino acid. These observations are consistent with the Michael addition mechanism, provided that reversal of the addition reaction is catalyzed by strong acid. Thus, the reduction of the aldehyde moiety would preclude reversibility and, thereby, lead to formation of an acid-stable derivative.

In the HPLC system used, *o*-phthaldialdehyde derivatives of the reduced forms of the 4-hydroxynonenal–histidine and 4-hydroxynonenal–lysine adducts can be separated from all other normal amino acids. As shown in Fig. 2, the histidine derivatives appear as three separate

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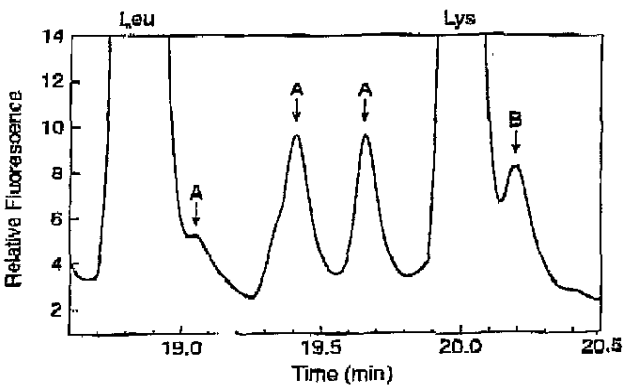

FIG. 2. Separation of 4-hydroxynonenal adducts by HPLC. The enzyme (1 mg/ml) was incubated with 2 *mM* 4-hydroxynonenal in 50 *mM* sodium phosphate buffer (pH 7.2) for 2 hr at 37°. After reduction with $NaBH_4$ reaction mixtures were hydrolyzed, and the amino acid composition was analyzed by HPLC following derivatization with OPA. Peaks A correspond to the 4-hydroxynonenal–histidine adducts. Arrow B indicates the 4-hydroxynonenal–lysine peak. The peaks marked Leu and Lys correspond to the OPA derivatives of leucine and lysine, respectively.

peaks (isomers?) which elute between leucine and lysine at 19.05, 19.4, and 19.7 min, whereas the lysine adduct elutes just after lysine at 20.2 min. By integration of the areas under the peaks and comparison with the areas of amino acid standards, the amounts of histidine and lysine 4-hydroxynonenal adducts can be quantitated.

Discussion

We found that the major product formed in the reaction of 4-hydroxynonenal with α-*N*-acetyllysine or poly(L-lysine) is the Michael addition product, namely, a secondary amine produced by addition of the ε-amine group of lysine to the double bond (C-3) of 4-hydroxynonenal.[13] For many of 4-hydroxynonenal-modified proteins tested (glyceraldehyde-phosphate dehydrogenase, insulin, bovine serum albumin, low-density lipoproteins), the number of 4-hydroxynonenal–histidine residues detected by these procedures was almost exactly equal to the number of histidine residues that disappeared.

However, the number of lysine–hydroxynonenal and cysteine–hydroxynonenal adducts that can be detected by HPLC assay of the α-phthalaldehyde derivatives and the Raney nickel treatment, respec-

tively, is often considerably lower than the number of these residues that disappeared on treatment with 4-hydroxynonenal. For example, reaction of 4-hydroxynonenal with glyceraldehyde-3-phosphate dehydrogenase under the conditions described here led to the modification of 5 histidine, 3.5 lysine, and 2.5 cysteine residues.[17] By means of the Raney nickel procedure, only 17% of the modified cysteine could be attributed to a simple Michael addition reaction, whereas 90 and 28%, respectively, of the histidine and lysine residues were present as simple Michael addition products, as determined by HPLC of the *o*-phthalaldehyde derivatives of $NaBH_4$-treated acid-hydrolyzed samples. It was proposed that the poor recovery of lysine and cysteine residues might be due to secondary reactions in which the aldehyde groups of some primary Michael addition products react with proximal lysine residues to form Schiff base cross-links, which would be stabilized by reduction with $NaBH_4$.[17] This possibility is supported by (1) the observation that the number of cysteine plus histidine residues that could not be accounted for as Michael addition products is equal to the number of lysine residues that could not be accounted for and (2) by the appearance of protein conjugates which sodium dodecyl sulfate (SDS) gel electrophoresis exhibited molecular weights about two times that of the native subunit.[17]

Acknowledgments

We thank Dr. H. Esterbauer (University of Graz) for the generous gift of 4-hydroxynonenal diethylacetal. We also thank Dr. R. L. Levine and B. S. Berlett (National Institutes of Health) for advice on amino acid analysis.

[41] Measurement of Protein Thiol Groups and Glutathione in Plasma

By MIAO-LIN HU

Introduction

Essentially all of the plasma sulfhydryl (SH) groups are protein associated.[1,2] Albumin is the most abundant plasma protein (40–60 mg/ml) and

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